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JNK-ATF-2 inhibits thrombomodulin (TM) expression by recruiting histone deacetylase4 (HDAC4) and forming a transcriptional repression complex in the *TM* promoter

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ABSTRACT

Thrombomodulin (TM) is an important vascular protective molecule that has anticoagulant, anti-inflammatory and anti-apoptotic properties. TM is downregulated in many thrombotic and vascular diseases. However, the mechanisms responsible for TM suppression are not completely understood. In this study, we investigated the mechanism involved in fatty acid-induced suppression of TM expression in human aortic endothelial cells. We found that palmitic acid inhibited TM expression through the JNK and p38 pathways. ATF-2, a JNK and p38 target transcription factor, was involved in the suppression. ATF-2 can bind to the *TM* promoter, recruit HDAC4 and form a transcriptional repression complex in the promoter, which may lead to chromatin condensation and transcriptional arrest. This study provides novel insight into TM down-regulation by stress signaling pathways.

Structured summary:
MINT-7555703, MINT-7555712: *HDAC4* (uniprotkb:P56524) *physically interacts* (MI:0915) with *ATF-2* (uniprotkb:P15336) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Endothelium plays an active role in regulating pro-coagulation and anti-coagulation balance by generating several active regulatory molecules, such as von Willebrand factor (vWF), thrombomodulin (TM), tissue plasminogen activator (t-PA), and plasminogen activator inhibitor (PAI-1) [1,2]. Among these factors, TM-protein C pathway is a major physiological anticoagulation system of the endothelium [3]. Endothelial dysfunction can cause coagulation dysregulation and promote vascular thrombosis.

TM, a glycoprotein on the surface of endothelial cells, is a key factor in protein C activation [3]. When bound to thrombin, TM triggers the activation of protein C by facilitating the conversion of circulating protein C to activated protein C (APC). APC can inhibit coagulation by degrading VIIIa [4] and factor Va [4–6] and enhance fibrinolysis by inactivating PAI-1 [7]. TM plays a key role in anticoagulation. Mutation or down-regulation of TM promotes [8,9], while overexpression of TM prevents [10] arterial thrombosis. In addition, TM functions as an anti-inflammatory and anti-apoptotic molecule. It has been shown that TM inhibits inflammatory response [11–14] and blocks cell apoptosis [15–17]. TM is down-regulated in vascular diseases including atherosclerosis [18], and TM is negatively regulated by inflammatory factors [19–21], wall tension [22,23] and oxidized lipids [24–26]. However, the mechanisms involved in the down-regulation of TM expression are not completely understood.

Stress signaling JNK and p38 pathways are activated in many cardiovascular diseases including atherosclerosis and are involved in pathophysiological changes in these conditions [27,28]. JNK and p38 signaling pathways are activated by metabolic stress [29] and inflammatory factors [30,31]. We previously showed that JNK and p38 can be activated by free fatty acids (FFA) and were involved in vascular insulin resistance [29], it is interesting to know whether activation of these pathways by FFAs also affects TM expression.

In this study, we examined the effects of activation of JNK and p38 by FFAs on TM regulation and investigated the mechanisms involved.

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2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs) (Cell Applications, San Diego, CA) were grown in endothelial cell basic medium (EBM) containing 2% FBS, FGF-2, VEGF, IGF-1, EGF, ascorbic acid, GA-1000, hydrocortisone, and heparin. HAECs, of 5–9 passages, were plated on six-well plates, and then treated with palmitic acid or transfected with siRNAs.

2.2. Preparation of fat acid–albumin complexes

HAECs were treated with 0.5% BSA alone or different concentrations of saturated palmitic acid (PA, also known as hexadecanoic acid) for 24 h. Preparation of PA was carried out as previously [29]. Briefly, PA was dissolved in ethanol at 200 mM, and then combined with 10% FFA-free low endotoxin BSA to final concentrations of 1–5 mM. The pH of all solutions was adjusted to approximately 7.5, and the stock solutions were stored at −20 °C. Control solution containing ethanol and BSA was prepared similarly. Working solutions were prepared fresh by diluting stock solution (1:10) in 2% FCS-EBM. The final 1% BSA was consistent in all FFA medium, whereas the FFA/BSA ratio varied with the FFA concentrations.

2.3. siRNA transfection

Negative control siRNA or specific p38 siRNA, JNK siRNA and ATF-2 siRNA were purchased from Ambion (Austin, TX). HAECs were transfected with negative control or specific siRNAs using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transfected cells were treated with palmitic acid for 24 h.

2.4. Western blotting

Cell lysate from treated HAECs were prepared as described previously [29,31]. Protein concentration was measured using a Bio-Rad Protein Assay Reagent kit (Bio-Rad, Hercules, CA). The cell lysates were subjected to SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked, incubated overnight with primary antibody, washed, and then incubated with the secondary horseradish peroxidase-labeled antibody. Signal detection was performed with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The data shown was representative of three separate experiments.

2.5. RNA extraction and real-time quantitative PCR

Total RNA was extracted from HAECs with Trizol (Invitrogen). Signal-strand cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad). Semi-quantitative real-time PCR was performed with iCycler iQ real-time PCR detection system (Bio-Rad). The primers for human TM mRNA were as follows: forward: 5′-CCGATGTC ATTTCCTTGCTA-3′; reverse: 5′-GTTGTCTCCCGTAACCCACT-3′. The mRNA levels were acquired from the value of the threshold cycle (Ct) of *TM* normalized against the Ct of β-actin.

2.6. Chromatin immunoprecipitation assay

The ChIP assay kit (Upstate) was used as previously described [29,31]. In brief, treated HAECs were first incubated with 1% formaldehyde at 37 °C for 15 min to cross-link DNA–protein complexes. Cells were then rinsed and lysed. Cell lysates were sonicated and centrifuged to produce chromatin fragments. The supernatants were pre-cleared with a mixture of salmon sperm DNA/protein A/protein G, followed by immunoprecipitation with antibody-protein A-agarose slurry (IgG served as the negative control). The immunocomplex beads were then washed sequentially with low salt-wash buffer, high salt-wash buffer, LiCl wash buffer, and TE buffer. The immunocomplex was eluted with elution buffer. The eluted immunocomplex and the inputs were incubated with 200 mM NaCl at 65 °C overnight to reverse the cross-link, and then incubated with proteinase K to digest the remaining proteins. The DNA was recovered by extraction with the phenol/chloroform/isoamyl alcohol mixture. The immunoprecipitated DNA was used as a template for PCR. The PCR products were separated by 1.5% agarose gel. The primers used for the AP-1 binding site in the 5′-flanking region of the human *TM* gene were: forward primer 5′-TAAA CAGTTTGCCTCTCACC-3′ and reverse primer 5′-TCCTGTGGATGGG CAGGGTG-3′.

2.7. Immunoprecipitation

Immunoprecipitation was conducted as described previously [32,33].Treated cells were lysed for 60 min in ice-cold extraction buffer containing 50 mM Tris–Cl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 50 mM β-glycerophosphate, and a protease inhibitor mixture (Amersham Biosciences). For immunoprecipitation, cleared cell lysates were incubated with the appropriate antibody precoupled to protein A/G-agarose beads (Santa Cruz Biotechnology) at 4 °C overnight. The beads were washed twice with extraction buffer and twice with extraction buffer containing 0.5 M LiCl. Proteins were eluted directly in SDS sample buffer for Western blot analysis.

2.8. Statistical analysis

Data are presented as mean ± S.E.M. of three independent experiments. One-way ANOVA was used to analyze the differences among groups. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Free fatty acids suppressed TM expression in HAECs

We first examined whether palmitic acid (PA) regulated the expression of TM in HAECs. HAECs were incubated with different concentrations of PA for 24 h. Western blotting showed that PA significantly suppressed the expression of TM in a dose-dependent manner (Fig. 1A). Furthermore, PA significantly inhibited the expression of TM mRNA (Fig. 1B), indicating that PA may suppress TM expression at the transcriptional level.

3.2. JNK and p38 stress pathways were involved in PA's inhibitory effect on TM expression

We next determined whether JNK and p38 pathways were involved in PA's inhibition of TM expression. The activation of these two pathways was examined. Consistent with previous observation [29], JNK and p38 were activated by PA in a dose-dependent manner (Fig. 2A). Importantly, silencing JNK and p38 with specific siRNAs reversed PA-induced TM suppression (Fig. 2B), indicating that JNK and p38 pathways mediated PA-induced inhibition of TM expression.

3.3. ATF-2 was involved in JNK and p38-mediated TM suppression

We further studied the mechanisms by which JNK and p38 mediated PA-induced down-regulation of TM mRNA. JNK and

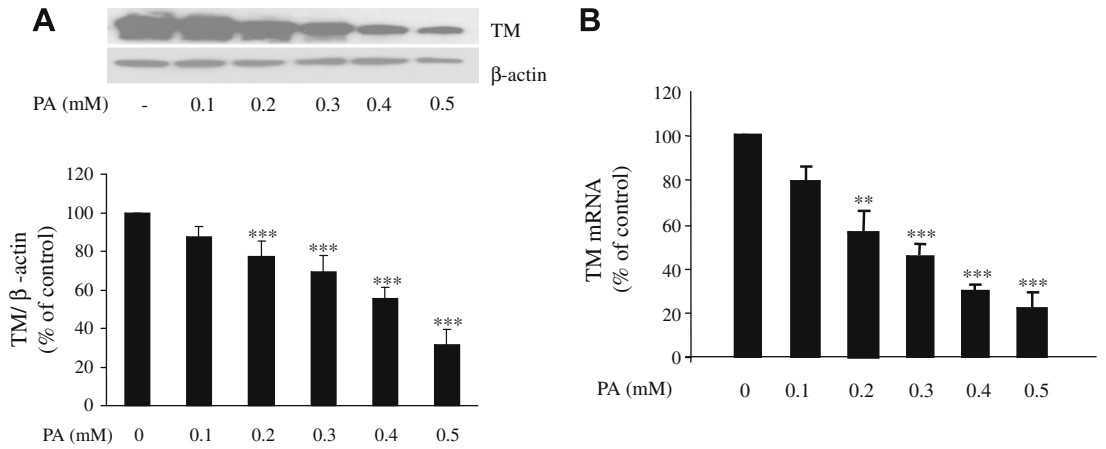

Fig. 1. Palmitic acid (PA) inhibited thrombomodulin (TM) expression in human aortic endothelial cells (HAECs). HAECs were treated with different concentrations of PA for 24 h. (A) TM protein expression was examined by Western bolt using anti-TM antibody. The relative levels of protein were compared and expressed as percentages of the control. Representative blots and quantitative analyses from three independent experiments are shown. ***$P < 0.001$ versus the no-treatment control. (B) Relative TM mRNA was analyzed by RT-PCR and normalized with β-actin mRNA. The relative levels of mRNA were compared and expressed as the percentage of the control. Results are expressed as means ± S.E.M. ($n = 3$). **$P < 0.01$, ***$P < 0.001$ versus no PA control.

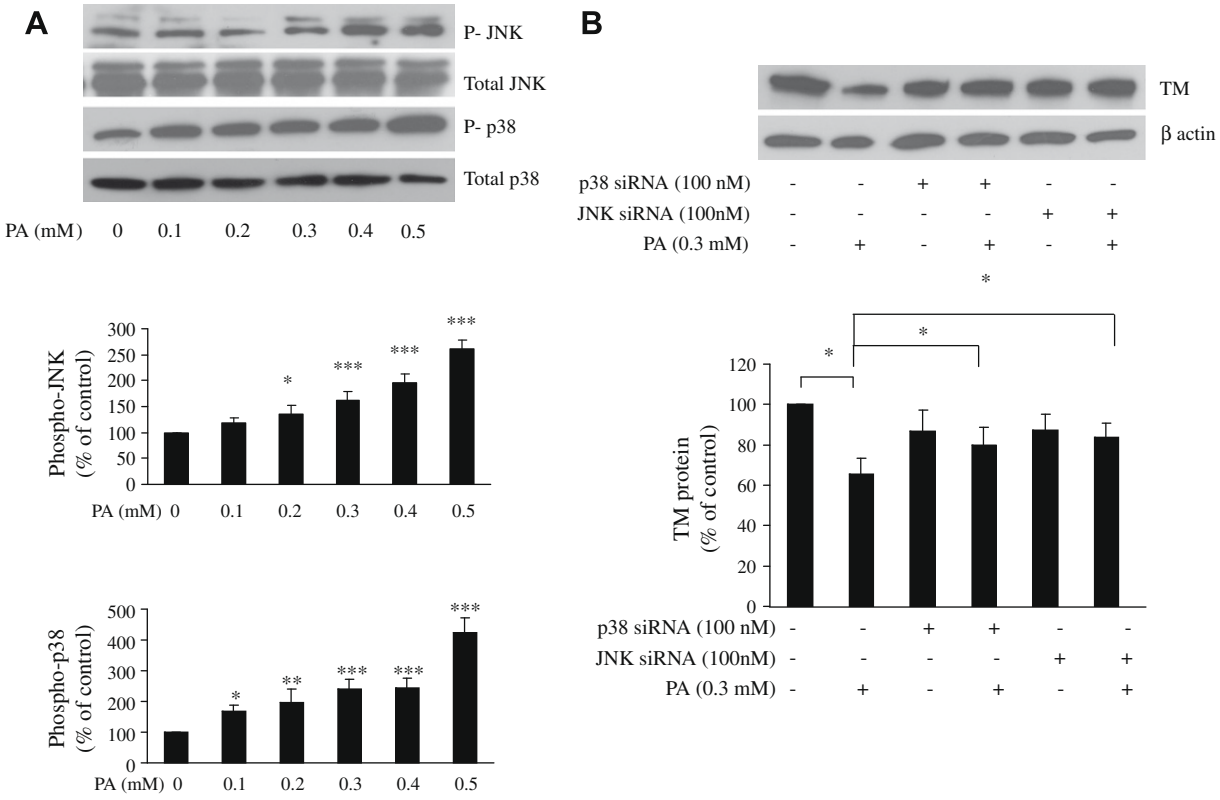

Fig. 2. JNK and p38 pathways mediated PA's inhibitory effect on TM expression. (A) Activation of JNK and p38 by PA. HAECs were treated with different concentrations of PA for 24 h. The phosphor-JNK and total-JNK as well as phosphor-p38 and total-p38 were detected by Western blot. (B) JNK and p38 pathways mediated PA's inhibitory effect on TM expression. HAECs were transfected with negative control siRNA, or JNK siRNA or p38 siRNA followed by incubation with PA for 24 h. TM expression was examined by Western blot. The relative levels of protein were compared and expressed as percentages of the control. Representative blots and quantitative analyses from three independent experiments are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the no-treatment control, or as indicated.

p38 are involved in gene regulation by activating an array of transcriptional factors. The most important target transcriptional factors are activating protein-1 (AP-1), a group of transcription factors including Jun, Fos, ATF and Maf. We have previously shown that PA can activate ATF-2, which mediates p38-induced upregulation of PTEN [29]. We therefore investigated whether ATF-2 was involved in the inhibition of TM transcription. ATF-2, a member of the ATF/CREB family of transcription factors, binds to the ATF/CREB site 5′-TGACGTCA-3′ and the AP-1 site 5′-TGACTCA-3′ [34]. The promoter region in the *TM* gene contains several putative AP-1 half binding sites, including agTGACGgatt at −1288/−1277, gcTGACTcgct at −1026/−1016, and ccTGACAgtgt at −939/−929 (Fig. 3A). We first examined whether ATF-2 can bind to the *TM* promoter. Using the chromatin immunoprecipitation (ChIP) assay, with an ATF-2 antibody for immunoprecipitation of the DNA–protein complex and subsequent PCR to detect associated DNA, we

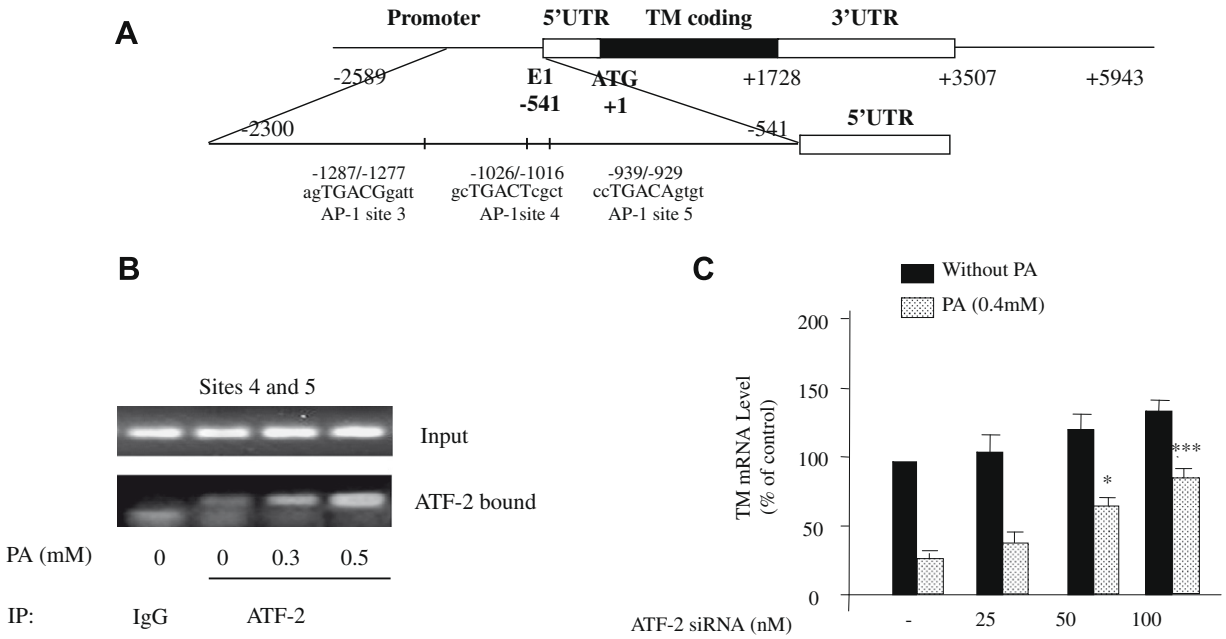

Fig. 3. Involvement of ATF-2 in PA-induced down-regulation of thrombomodulin expression. (A) Depiction of AP-1 sites in the *TM* promoter. (B) ATF-2 bound to *TM* promoter and PA increased binding of ATF-2 to AP-1 sites in the *TM* promoter. HAECs were treated with PA for 24 h. Protein–DNA complex cross-linked by formaldehyde was immunoprecipitated using anti-ATF-2 antibody, and specifically bound AP-1 sites were detected by PCR. (C) Involvement of ATF-2 in PA-induced down-regulation of thrombomodulin mRNA. HAECs were transfected with negative control siRNA or ATF-2 siRNA and then treated with or without PA for 24 h. Relative TM mRNA was analyzed by RT-PCR and normalized with β-actin mRNA. The relative levels of mRNA were compared and expressed as the percentage of the control. Data represent the mean ± S.E.M. ($N = 3$). $^{*}P < 0.05$, $^{***}P < 0.001$ versus PA with scrambled siRNA.

observed that ATF-2 can bind to the *TM* promoter at the gcTGACTcgct (−1026/−1016) and ccTGACAgtgt (−939/−929) sites (Fig. 3B). Importantly, the binding was significantly increased by PA treatment, indicating that binding of ATF-2 to *TM* promoter may be involved in PA-induced suppression of TM transcription. Indeed, when HAECs cells were transiently transfected with ATF-2-specific siRNAs before treated with PA, PA-induced inhibition of *TM* expression was significantly prevented (Fig. 3C). These data support a critical role for ATF-2 transcription factor in PA-induced down-regulation of TM transcription.

3.4. Recruitment of HDAC4 and formation of AFT-2/HDAC4 transcription repressor complex in the TM promoter

We finally investigated how the transcription factor ATF-2 can bind to the *TM* promoter and inhibit gene expression; one possible mechanism is chromatin remodeling. Transcription factors can bind to a promoter and form a transcription repressor complex that recruits co-repressors such as HDACs, which deacetylate histone, condense chromatin, and thereby inhibit gene transcription. To test whether this mechanism applies in ATF-2-mediated transcription repression of the *TM* gene, we first identified HDACs that can bind to the *TM* promoter. Using the ChIP assay, we found that PA treatment significantly increased HDAC4 binding to the *TM* promoter (Fig. 4A). We then determined whether ATF-2 associates with HDAC4 in vivo. Using a coimmunoprecipitation assay, we observed that ATF-2 was associated with HDAC4 and that the association was increased by PA treatment (Fig. 4B), indicating that HDAC4 recruitment to the *TM* promoter may be mediated, at least in part, by ATF-2. Finally, we used the double-chip assay to determine if ATF-2 and HDAC4 were in the same transcription repression complex in the *TM* promoter. The initial immunoprecipitation was conducted with an ATF-2 antibody, and the subsequent immunoprecipitation was done using the anti-HDAC4 antibody. The associated DNA in the immunocomplex was amplified by PCR. The double-chip assay (Fig. 4C) showed the *TM* promoter sequence can be recovered from the immunocomplexes precipitated by ATF-2 and HDAC4 antibodies, indicating the simultaneous association of ATF-2 and HDAC4 within the region of the *TM* promoter. Together, these results suggest that activated ATF-2 may recruit HDAC4 and form a transcription repression complex in the *TM* promoter.

4. Discussion

In the present study, we demonstrated that activated JNK and p38 were involved in palmitic acid induced inhibition of TM expression in HAECs. Their target transcription factor ATF-2 mediated the inhibition. ATF-2 can bind and recruit HDAC4 to the *TM* promoter, which may lead to histone deacetylation, chromatin condensation and transcription arrest (Fig. 4D).

Thrombin-TM-protein C pathway is an important anticoagulant system and TM is the key component in the system [6,14,35,36]. Downregulation of TM expression is associated with many thrombotic and vascular conditions [6,14,35,36]. TM can be negatively regulated by inflammatory factors [19–21] and oxidized lipids [24–26]. In the present study, we demonstrated that TM expression can be inhibited by palmitic acid in HAECs, a mechanism that may be implicated in the prothrombotic tendency in metabolic syndrome.

We further investigated the mechanisms involved. JNK and p38 pathways can be activated by stress signals and contribute to many pathological changes in cardiovascular diseases. Increasing evidence suggest that activation of these pathways may also be responsible for thrombosis dysregulation. It has been shown that p38 and JNK are involved in platelet activation and aggregation [37], upregulation of the expression of TF [38,39] and PAI-1 [40,41]. Recently, it has been shown that JNK and p38 mediated TNFα induced down-regulation of TM expression [42]. Consistent with this finding, our study demonstrated that JNK and p38

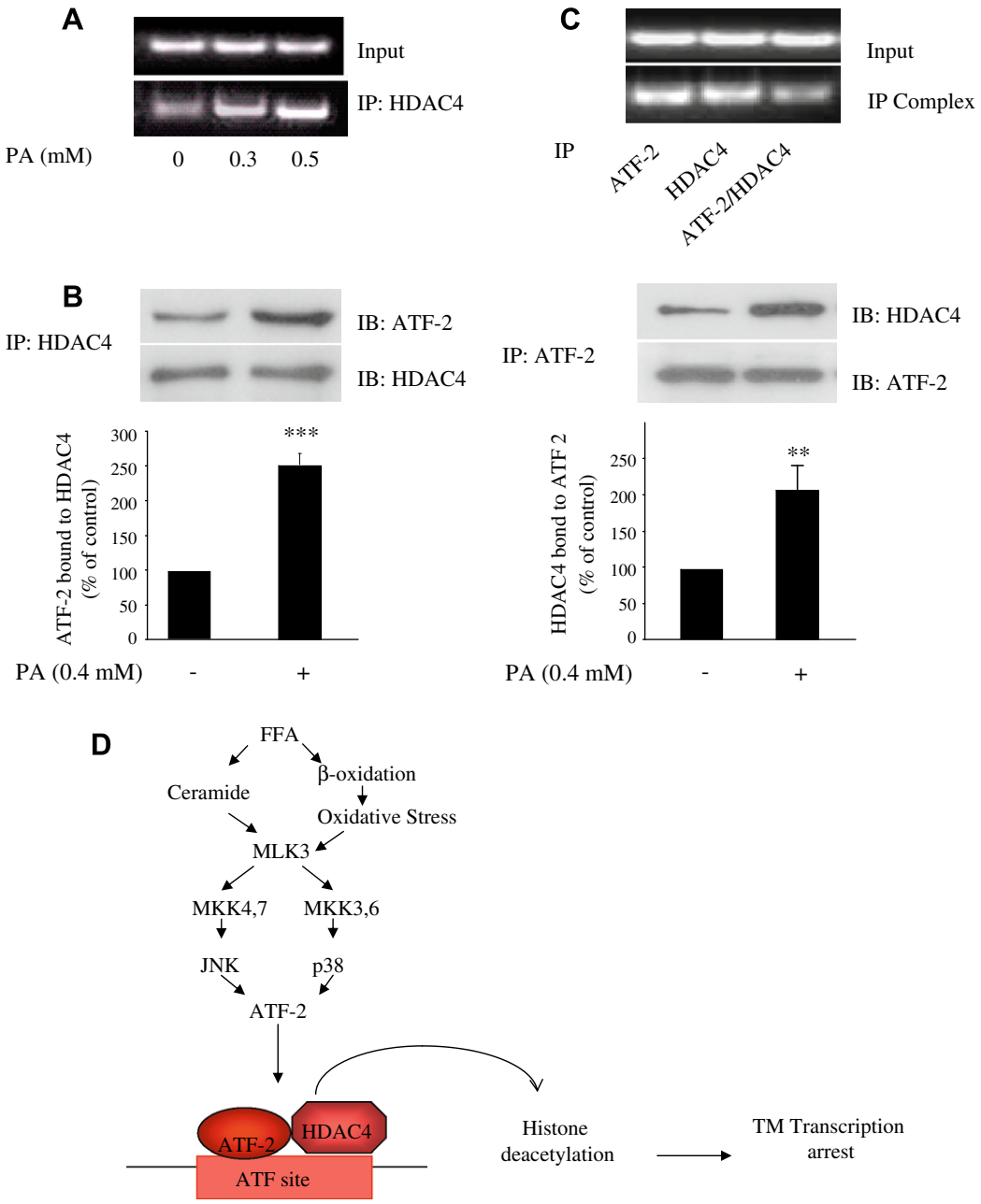

Fig. 4. Formation of AFT-2/HDAC4 transcription repressor complex in the *TM* promoter. (A) PA increased recruitment of HDAC4 to the *TM* Promoter. HAECs were treated with PA for 24 h. Protein–DNA complex was immunoprecipitated using anti-HDAC4 antibody. The AP-1 site in the DNA–protein complex was amplified by PCR. (B) Interaction between ATF-2 and HDAC4. ATF-2 and HDAC complex was immunoprecipitated with anti-ATF-2 antibody, and the presence of HDAC4 in the complex was detected by anti-HDAC4 antibody. Alternatively, the complex was immunoprecipitated with anti-HDAC4 antibody, and ATF-2 in the complex was detected by anti-ATF-2 antibody. The relative levels of protein were compared and expressed as percentages of the control. Representative blots and quantitative analyses from three independent experiments are shown. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. (C) Formation of AFT-2/HDAC4 transcription repressor complex in the *TM* promoter. HAECs were treated with PA for 24 h. Protein–DNA complex cross-linked by formaldehyde was first immunoprecipitated with anti-ATF-2 antibody and then with anti-HDAC4 antibody. The ATF/CREB site in the complex was specifically detected by PCR. (D) Proposed mechanisms of TM repression by stress signaling. FFAs activate JNK and p38 pathways, which activate target transcription factor ATF-2. After binding to the *TM* promoter, ATF-2 recruits HDAC4, which leads to histone deacetylation, chromatin condensation, and transcription suppression.

pathways were also involved in fatty acid induced suppression of TM expression. Thus, activation of JNK and p38 stress signaling pathways may play significant roles in the dysregulation of the TM-protein C system and the coagulation–anticoagulation imbalance.

How stress signaling down-regulates TM expression? ATF-2 is a downstream target transcription factor of JNK and p38 pathways. Previous study has reported that ATF-2 mediated LPS-induced TF expression [43] and may be involved in thrombosis dysregulation. Recently, it has been shown that shear stress induces expression of protective genes including TM, probably through inhibition of ATF-2 [44]. In this study, we showed that ATF-2 mediated stress signaling induced TM inhibition. ATF-2 bound to AP-1 binding sites in the *TM* promoter and negatively regulated TM transcription, a process that was enhanced by fatty acid treatment.

Furthermore, we examined how ATF-2 can bind to *TM* promoter and suppress gene expression. The transcription switch of a given gene is controlled by the coordinated activities of transcription

activator complexes and transcription repressor complexes. Transcription repressor complexes recruit co-repressors such as HDACs, which deacetylate histone, condense chromatin, and inhibit transcription. In contrast, transcription activator complexes recruit co-activators (i.e., CBP/p300), which acetylate histones, unwind chromatin, and thereby promote transcription. Here we showed that ATF-2 recruited HDAC4 and formed a transcription repressor complexes on *TM* promoter, which may lead to histone deacetylation and subsequent transcription repression.

Nevertheless, other inhibitory mechanisms on TM transcription may also exist. ATF-2 may inhibit transcription factors such as Sp-1 or coactivators such as CBP/p300, thus preventing the formation of the functional transcription activator complex in the *TM* promoter. Furthermore, TM can be controlled at different stages of its production. Although our study leads us to hypothesize that down-regulation of TM transcription may be an important mechanism for decreased TM levels, FFAs may suppress TM protein expression by reducing mRNA stability, inhibiting protein translation, or promoting protein degradation through an ubiquitin-proteasome pathway. Further studies will be necessary to define the detailed mechanisms for the dysregulation of the TM-APC system by metabolic stress and stress signaling pathways.

In summary, FFAs inhibit the expression of TM in HAECs. The regulation is mediated by JNK and p38 pathways, which induce the ATF-2/HDAC4 transcription repressor complex in the *TM* promoter. This study provides novel insights into the molecular mechanisms of stress signaling-induced suppression of TM expression.

5. Disclosure

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.048.

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